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Wnt is necessary for mesenchymal to epithelial transition in colorectal cancer cells.

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Abstract

Background: Metastasis underlies most colorectal cancer mortality. Cancer cells spread through the body as single cells or small clusters of cells that have an invasive, mesenchymal, non-proliferative phenotype. At the secondary site, they revert to a proliferative ‘tumor constructing’ epithelial phenotype to rebuild a tumor. We previously developed a unique *in vitro* 3D model, called LIM1863-*Mph*, which faithfully recapitulates these reversible transitions that underpin colorectal cancer metastasis. Wnt signaling plays a key role in these transitions and is initiated by the coupling of extracellular Wnt to Frizzled (FZD). Using the LIM1863-*Mph* model system we demonstrated that the Wnt receptor FZD7 is necessary for mesenchymal to epithelial transition (MET). Here we investigate the role of Wnt ligands in MET. **Results:** Wnt secretion is dependent on palmitoylation by the enzyme Porcupine (PORC). A PORC inhibitor (IWP2) that prevents the secretion of Wnt proteins including Wnt2B, blocked the epithelial transition of mesenchymal LIM1863-*Mph* cells. Wnt gene array analysis identified several Wnts that are upregulated in epithelial compared to mesenchymal LIM1863-*Mph* cells, suggesting these ligands in MET. Wnt2B was the most abundant differentially expressed Wnt gene. Indeed, recombinant Wnt2B could overcome the IWP2-mediated block in epithelial transition of mesenchymal LIM1863-*Mph* cells.

Conclusion: Wnt2B co-operates with Frizzled7 to mediate MET in colorectal cancer.

Introduction

Colorectal cancer (CRC) is a major health problem worldwide and, similar to other forms of cancer, it is much harder to treat once it has spread to other organs in the body. A necessary step in the genesis of most CRCs is deregulation of the β -catenin dependent Wnt signaling pathway (Clevers and Nusse 2012), which occurs through genetic mutation of downstream pathway components, primarily the tumor suppressor gene *Adenomatous Polyposis Coli* (*APC*) (Fearon 2011). At a molecular level, the Wnt/ β -catenin pathway regulates the cytoplasmic and nuclear levels of β -catenin via a cytoplasmic destruction complex that includes APC, Axin and GSK3 β . Mutations of *APC* in CRC disrupts this regulation and β -catenin accumulates in the cytoplasm, translocates to the nucleus and leads to the constitutive activation of TCF/LEF/ β -catenin transcription of target genes (Korinek et al. 1997, van de Wetering et al. 2002). However, the level of nuclear β -catenin is variable in CRC despite activating mutations in the downstream components of the pathway (Brabletz et al. 1998, Brabletz et al. 2001), indicating additional regulation of the Wnt/ β -catenin pathway by the tumor environment (Vincan and Barker 2008).

The majority of CRCs are well differentiated forming organized tubular structures, yet they still metastasize, and therefore differentiation status is often a poor prognostic factor for this cancer. Both primary and secondary (metastases) well-differentiated tumors have clearly defined localized invasive areas where the tubular structures of the CRC is reorganized yielding tumor cell sheets and isolated tumor cells interspersed with the stroma (Brabletz et al. 1998, Brabletz et al. 2001). Histological detection of invasive areas in the tumor predicts poor outcome, independent of tumor staging (Ueno et al. 2014, Wong et al. 2003). Tumor cells engaged in tubular structures are well differentiated with membrane β -catenin (polarized epithelial cells) that stain with Ki-67. In contrast, cell division (Ki-67 staining) is decreased or undetected in de-differentiated (migratory, invasive, mesenchymal) tumor cells with intense cytoplasmic and nuclear β -catenin at invasive areas (Brabletz et al. 2001). Based on these phenotypic changes, de-differentiation at the invasive front is referred to as an epithelial to mesenchymal transition (EMT) (Kirchner and Brabletz 2000). Once in the secondary organs, the reverse transition occurs (MET) which re-instates the epithelial, proliferative phenotype. Notably, the pathology of primary and secondary tumors for most CRCs is the same (Brabletz et al. 2005a, Thiery 2002). Transitions between epithelial and mesenchymal states are thus dynamic and reversible. Presumably, this reversal of de-differentiated disseminated cells to an

epithelial phenotype is necessary to re-instate cell division so that the tumor structure can be re-built at the secondary site (Thiery 2002, Vincan 2004).

During metastasis cancer cells disseminate through the body as single cells or small clusters of cells and can remain ‘dormant’ for many years (MacDonald et al. 2002, Naumov et al. 2002). Moreover, disseminated cancer cells (mesenchymal, non-proliferating) evade conventional anti-cancer therapies, which in general target proliferating cells. The ability to eliminate these ‘dormant’ cancer cells is critical for curative treatment. Initiation of tumor growth at the secondary site is the rate-limiting step in metastasis (Chambers et al. 2002). Consequently, circumventing release from dormancy offers a further avenue for therapeutic intervention. However, the molecular mechanisms controlling early events of release from ‘dormancy’ are still largely unknown due to the complexity this presents for analysis *in vivo*. To overcome this limitation, we have established a unique *in vitro* morphogenesis culture system using the human colorectal cancer cell line LIM1863 (Whitehead et al. 1987). In this model system, LIM1863 cells growing as a two-dimensional (2D) monolayer undergo MET to form 3D highly organized, multicellular structures that resemble enclosed carcinoma tubules (termed organoids). We demonstrated cyclic transition from 2D to 3D and refer to this adapted cell line as LIM1863-*Mph* (morphogenetic) (Vincan et al. 2007a, Vincan et al. 2007b, Vincan et al. 2008). The dynamic phenotype transitions between 2D and 3D faithfully recapitulate the pathology of invasive (deconstructing tubular structures) and central (constructing tubular structures) areas of carcinoma tissues (Vincan et al. 2007a). We recorded nuclear β -catenin in the monolayer cells (low or undetectable Ki-67 staining), then a transient, short, sharp increase in nuclear β -catenin at the initiation of MET, just as the cells lift off the tissue culture plastic and become Ki-67 positive. This intense nuclear β -catenin rapidly subsides to undetectable levels as the organoids form, while Ki-67 staining is retained as all the organoid cells proliferate. Using RNAi-mediated knock-down in LIM1863-*Mph*, we demonstrated that the Wnt receptor Frizzled7 (FZD7) is necessary for MET (Vincan et al. 2007b). FZD7-mediated Wnt/ β -catenin signaling is known to orchestrate tissue “constructing” morphogenetic events during embryonic development (e.g. epithelial organization of somites (Linker et al. 2005)) and our findings indicate a similar role for FZD7 in the context of CRC (Vincan et al. 2007c).

However, the Wnt ligands that co-operate with FZD7 during the initiation of MET are not known. Here we show that Wnt is required for MET in the LIM1863-*Mph* model as inhibiting Wnt secretion blocks MET. Secretion of Wnt is dependent on porcupine (PORC) (van den

Heuvel et al. 1993), an enzyme in the endoplasmic reticulum that post translationally palmitoylates Wnts at a highly conserved serine residue (Lum and Clevers 2012, Madan et al. 2016). Inhibition of PORC with IWP2 (B. Chen et al. 2009, S. Chen et al. 2009) blocked MET. We identify the Wnts that are differentially expressed by mesenchymal and epithelial LIM1863-*Mph* cells using an array platform; and demonstrate that recombinant Wnt2B or the archetypical β -catenin activating Wnt, Wnt3a, can partially rescue MET when Wnt secretion is blocked by IWP2.

Results

Wnt expression and signaling in LIM1863-*Mph* cells

The LIM1863-*Mph* cells undergo cyclic transitions between monolayer and free-floating organoid clusters. The organoids are spheres of highly polarized epithelial cells around a central lumen (Vincan et al. 2007a, Vincan et al. 2007b), shown diagrammatically in Fig. 1A. Having identified a role for FZD7 in orchestrating MET in the LIM1863-*Mph* model (Vincan et al. 2007b), and given that the expression of FZD7 does not change during these transitions (data not shown), the next important step was to identify which Wnt genes were differentially expressed by monolayer and organoid cells, and thus could potentially co-operate with FZD7 in this process. To answer this question, we employed the Human Wnt Signaling Pathway Array (Qiagen). Total RNA was extracted from LIM1863-*Mph* monolayer and organoid cells, cDNA synthesized and assayed using the cDNA array. Relative expression was determined using HMBS as reference (Fig. 1B). Wnt2B, Wnt3 and Wnt10A were markedly up-regulated in the organoids compared to the monolayer cells; expression of Wnt4 and Wnt11 were relatively unchanged, while the other Wnt genes were expressed at very low levels. Looking at expression levels of Wnt/ β -catenin target genes (Fig. 1C), these were similar in both cell types with less than two-fold changes in expression. The differences in Wnt2B, Wnt3 (and Wnt3A albeit expressed at very low levels) and Wnt target gene expression identified by the array analyses were validated by independent qRT-PCR on a separate set of cell preparations (Fig. 2A & 2B). Wnt2B was the most abundant differentially expressed Wnt and was thus investigated further. Wnt2B expression in the LIM1863-*Mph* organoids was confirmed by immunofluorescence and confocal microscopy, in which we observed Wnt2B localized in puncta apical to the nuclei of LIM1863-*Mph* organoid cells (Fig. 2C). As shown previously

(Vincan et al. 2007a, Vincan et al. 2007b), *laminin- γ 2*, a marker for the invasive front (Hlubek et al. 2001) is very strongly up-regulated (~30 fold) in the mesenchymal cells compared to the mature organoids. Similarly, the EMT marker *Claudin1* (Suh et al. 2013) is also up-regulated in the mesenchymal cells (Fig. 2B).

Wnt is necessary for MET

Wnt2B functions to promote the activity of intestinal stem cells (Farin et al. 2012), which are also the cell-of-origin in colorectal cancer (Barker et al. 2009). Furthermore, we have also recently shown that Wnt2B co-operates and co-immunoprecipitates with Fzd7 in intestinal stem cells (Flanagan et al. 2015), and therefore we focused on the function of this Wnt in MET, as stem cell properties are often hijacked by cancer cells (Barker et al. 2009, Pheesse et al. 2014).

First, we investigated whether Wnt is necessary for MET. Wnts are secreted growth factors and require post-translational modification by PORC to be secreted (B. Chen et al. 2009, S. Chen et al. 2009, Lum and Clevers 2012, Madan et al. 2016, van den Heuvel et al. 1993). Inhibition of PORC enzyme activity has been shown to inhibit the secretion of Wnts, and therefore we used the PORC inhibitor IWP2 as a tool to inhibit Wnt secretion. We confirmed inhibition of Wnt secretion using Wnt3A-L-cells, a mouse fibroblast continuous cell line that was stably transfected with a Wnt3A and secretes Wnt3A into the supernatant (Willert et al. 2003). We collected conditioned medium from parental L-cells, and Wnt3A-L-cells with or without the PORC inhibitor IWP2 (5 μ M). These conditioned media were then added to parental L-cells. We showed by immunoblot that Wnt3a conditioned medium induced the accumulation of β -catenin and that this activity was lost when the PORC inhibitor IWP2 was added during preparation of the Wnt3A-conditioned medium, thus confirming that IWP2 is functioning to inhibit Wnt secretion in these cells (Fig. 3A).

Next, monolayer organoids were treated with 5 μ M IWP2 as they were just starting to re-organize to form organoids i.e. undergoing MET. Inhibition of PORC blocked MET and the monolayer patch had a “cotton wool” appearance with no budding organoids, consistent with a mesenchymal phenotype (Vincan et al. 2007b). In contrast, numerous generating organoids were observed in the carrier-treated monolayer patch (Fig. 3B). Expression of the mesenchymal markers, *laminin- γ 2* and *Claudin1* remained high in the IWP2 treated cells and was increased

approximately two fold when compared to the control DMSO treated cells, confirming their mesenchymal phenotype (Fig. 3C). The expression of Wnt target genes was relatively comparable between the IWP2 treated and control cells (Fig. 3C). This is consistent with our observation that these genes are relatively unchanged when mesenchymal and epithelial LIM1863-*Mph* cells are compared (Fig. 1C).

Wnt2B and Wnt3A function in MET

Following the demonstration that inhibiting Wnt secretion blocked MET, we next investigated if addition of recombinant Wnts could rescue/restore MET. To better visualize the changes in the morphology during the MET process, the cells were seeded into chamber slides and stained for either E-cadherin or ZO-1, to demonstrate the cell architecture. Cultures were treated with DMSO, 5 μ M IWP2 or with 5 μ M IWP2 with either Wnt2B (100 ng/ml) or the archetypical β -catenin-activating Wnt, Wnt3a (100 ng/ml) (Willert et al. 2003). The E-cadherin stained DMSO treated cultures show that organoids were re-assembling because the monolayer cell patches have “holes”, i.e. areas where the cells are lifting off (arrows) and starting to re-organize themselves into organoids (indicated by the circles in Fig. 4A). In contrast, the IWP2 treated cells remain a tight patch and the E-cadherin staining does not distinguish any organoid formation or cells lifting off the tissue culture plastic (Fig. 4B). Adding recombinant Wnt2B (Fig. 4C) or Wnt3A (Fig. 4D) restores organoid assembly with “holes” observed in the monolayer patches, indicating the cells are lifting off and re-organizing into organoids. The ZO-1 staining shows the central lumen of the organoids being assembled in the DMSO treated cultures (Fig. 5A indicates one example of assembling organoids). These clusters of apical ZO-1 staining are not observed in the cultures treated with IWP2 (Fig. 5B). In contrast, the cluster of apical ZO-1 was clearly evident when recombinant Wnt was added to the IWP2-treated cultures (Fig. 5C & 5D). We have previously demonstrated that FZD7 is required for MET (Vincan et al. 2007b) and binds to Wnt2B (Flanagan et al. 2015). Thus, our collective data indicates that FZD7 transmits signals from Wnt2B to orchestrate MET in this model of colorectal cancer morphogenesis (Fig. 6).

Discussion

In CRC, aberrant activation of the Wnt/ β -catenin signaling pathway leads to the accumulation of β -catenin in the nucleus and thus the hyper-activation of β -catenin-TCF/LEF gene transcription. Indeed, the over-activation of the Wnt signaling pathway, caused by mutations to some of its intracellular components, is found in nearly all CRCs, both sporadic and familial (Kinzler et al. 1991). Most notable is the tumor suppressor gene *APC*, which is found to be mutated in approximately 80-90% of CRCs. However, in addition to these *APC* mutations, the over-expression of certain upstream signaling components can regulate Wnt signaling activity, irrespective of the downstream pathway-activating mutations (Caldwell et al. 2004, Suzuki et al. 2004, Ueno et al. 2009, Vincan et al. 2007b, Vincan et al. 2005). Importantly, additional Wnt signaling underlies malignant progression as the acquisition of invasive capabilities and metastasis relies on dynamic reversible processes, EMT and MET (Thiery 2002). Thus, it is of great importance to understand how these processes are coordinated and regulated, as it may provide novel avenues to block metastasis, which is ultimately the cause of cancer death. The results presented in this study demonstrate the potential of blocking the interaction between Wnt and FZD7, as a therapeutic target to prevent MET and cancer recurrence (Vincan and Barker 2008).

Although additional signaling by the Wnt-FZD receptor complex was initially considered unnecessary in CRC, it is now accepted that the upstream components participate in the initiation and progression of CRC. This is based on several lines of evidence. Expression of nuclear β -catenin in early adenoma is not as strong as it is in later stages (Phelps et al. 2009a, Phelps et al. 2009b). Also, naturally occurring inhibitors of Wnt-FZD interaction, the secreted Frizzled related proteins (sFRPs), are *bona fide* tumor suppressors in CRC (Caldwell et al. 2004, Suzuki et al. 2004), suggesting that the additional modulation of the β -catenin-dependent Wnt signaling pathway is via the upstream components of the pathway. Furthermore, epigenetic silencing of sFRP can occur before truncating mutations in *APC* (Suzuki et al. 2004), indicating that aberrant signaling from the Wnt-FZD complex may precede aberrant signaling due to truncation of *APC*. Furthermore, both Wnts (Dimitriadis et al. 2001, Katoh et al. 1996, Kirikoshi et al. 2001, Ru et al. 2008) and FZDs (He et al. 2011, Holcombe et al. 2002, Sagara et al. 1998, Ueno et al. 2008, Vincan 2004, Vincan et al. 2007b) are over-expressed in CRC tissues and cell lines and can additionally modulate the β -catenin-dependent Wnt signaling pathway (Ueno et al. 2009, Vincan et al. 2007b, Vincan et al. 2005), despite mutation to *APC* or *CTNN1* (the gene that codes for β -catenin). Notably, Wnt signaling appears to be constrained

in CRC cells [reviewed in (Pheesse et al. 2016)], giving way to bursts of localized increased signaling as occurs at the invasive front (Brabletz et al. 2005b) where aberrant activation of the Wnt pathway can induce EMT in tumor cells (Kim et al. 2002, Mariadason et al. 2001, Muller et al. 2002). Our collective data in the LIM1863-*Mph* model indicates that the initiation of MET is associated with a brief burst of even higher Wnt/ β -catenin signaling than that seen in the dormant mesenchymal cells at the invasive front (Brabletz et al. 2001, Vincan et al. 2007b).

Using the LIM1863-*Mph* model system, we demonstrated a requirement for FZD7 in MET that is necessary to assemble the organoids (Vincan et al. 2007b), but the question of which Wnt ligand was signaling via FZD7 remained unanswered. There are 19 mammalian Wnts and here we investigated which of these Wnt ligands interacts with FZD7 to activate MET. First, we employed a gene array and identified Wnt2B, Wnt3 and Wnt10A as differentially expressed by mesenchymal and organoid LIM1863-*Mph* cells. Next, we demonstrated a requirement for Wnt in the assembly of organoids by demonstrating that inhibition of Wnt secretion using IWP2 blocked MET. We chose to further investigate the role of Wnt2B in the LIM1863-*Mph* cells as we had identified this Wnt as a signaling partner of FZD7 in intestinal stem cells (Flanagan et al. 2015) and Wnt2B can activate β -catenin-dependent Wnt signaling (Goss et al. 2009). Furthermore, although it has been suggested that RNAi-mediated inhibition of PORCUPINE can suppress the growth of breast cancer cells in a Wnt independent mechanism, this did not phenocopy treatment with IWP-2 (cells did not respond to IWP-1 or IWP-2) (REF TO ADD; Covey et al, PLoS One, 2012, PLoS ONE 7(4): e34532.). . Indeed, we demonstrate that recombinant Wnt2B (or the archetypical β -catenin-activating Wnt, Wnt3A (Willert et al. 2003)) could restore MET when Wnt secretion was inhibited in the monolayer LIM1863-*Mph* cells. Notably, Wnt2B mediated β -catenin-dependent Wnt signaling is implicated during the morphogenesis of renal tubes (Iglesias et al. 2007), which is one of the key MET events during development. Thus a role for Wnt2B in promoting the formation of glandular structures in CRC is consistent with its role during development. Furthermore, Wnt2B was also one of the first Wnts demonstrated to be over-expressed in CRC cell lines (Bafico et al. 2004, Katoh et al. 1996); Notably, Wnt3 is also overexpressed in colorectal tumors (Voloshanenko et al. 2013). Thus the role we identify for Wnt2B, and potentially Wnt3, are consistent with over-expression reported for these Wnts in colorectal cancer.

Wnt3 produced by Paneth cells in the epithelium is essential for intestinal stem cells (Sato et al. 2011, Sato et al. 2009). In the absence of Wnt3, Wnt2B can compensate (Farin et al. 2012). More recently, it was shown, that high levels of Wnt2B are predominantly expressed in Gli1 positive sub-epithelial mesenchymal cells, which are adjacent to the crypts (Valenta et al. 2016). This localization of Wnt2B is consistent with previous reports showing Wnt2b expression in the mesenchyme of mouse intestine (Gregorieff et al. 2005). Collectively, these studies identify Wnt2B and Wnt3 as intestinal stem cell Wnts. Intriguingly, the data presented here indicates that these stem cell Wnts also function in colorectal cancer morphogenesis indicating that the stem cell Wnts, like FZD7, feature in colorectal cancer. Notably, we demonstrated that FZD7 stem cell function can be partially compensated by closely related FZD genes, FZD1 and FZD2 (Flanagan et al. 2015), however, the same compensatory mechanism does not appear to occur in cancer as knock-down of FZD7 blocks MET (Vincan et al. 2007b), and inhibiting FZD7 function with a dominant-negative receptor ectodomain has potent antitumor activity in colorectal cancer (Vincan et al. 2005) and other cancers such as liver cancer [reviewed in (Vincan and Barker 2008)].

Colorectal cancer (CRC) is a major health problem worldwide that is exacerbated by an aging population. CRC can effectively be cured if it remains localized and is surgically resected, however cancer cells often spread to other organs with metastases underlying most CRC mortality. Clearly better strategies are needed to stop tumor recurrence, which will ultimately lead to better management and treatment of advanced CRC, and consequently, increase patient survival. Furthermore, reversible EMT and MET underpins metastasis of other solid tumors (Ocana et al. 2012), thus the mechanisms for MET reported here might also be relevant to other forms of cancer.

Experimental Procedures

Cell lines, cell culture and live cell imaging

Cells lines were either purchased from ATCC (HEK293), provided by the Ludwig Institute for Cancer Research (LIM1863) (Whitehead et al. 1987) or the Nusse laboratory (parental L-cells and Wnt3a-Lcells) (Willert et al. 2003). The adapted LIM1863-*Mph* line has been previously described (Vincan et al. 2007a, Vincan et al. 2008). Cells were cultured in

RPMI 1640 medium (GIBCO, 11875-119), supplemented with 10% heat inactivated fetal calf serum (FSC, Chemicon International, Embryo Max FCS, ES qualified ES-009-A), 20 mM HEPES, Glutamine and antibiotics (Penicillin/Streptomycin). The LIM1863-*Mph* cells were cultured in rich medium with additional supplements [1 µg/mL hydrocortisone (Solu-Cortef for injection), ~0.9-1µg/mL insulin (Insulin for injection, 100U/mL, from Pharmacy) and 10⁻⁴M 1-thioglycerol (Sigma, M6145)]. The cells were incubated at 37°C in a humidified atmosphere containing 10% CO₂.

To plate the LIM1863-*Mph* so that they preferentially form monolayer patches, adherent LIM1863-*Mph* cells were detached with Trypsin/EDTA solution [0.05% (w/v trypsin 1:250, 0.02% (w/v) EDTA, pH7.0] and seeded at 5 x 10³ cells/cm² tissue culture well surface area as previously described (Vincan et al. 2008).

Differential interference contrast (DIC) images of LIM1863-*Mph* cultures were taken on a Nikon Ti-E microscope using either a 4× Plan Fluor NA 0.13 objective or a 10× Plan Fluor NA 0.3 objective. A focal stack of images was collected 10µm apart and processed through the “Best Focus” function of MetaMorph v7.7.7 (Molecular Devices) to generate the final image (Vincan et al. 2007b).

Detection of β-catenin by immunoblot

L-cells were seeded in replicate wells treated with conditioned medium (CM) harvested from the L-cell parental line, or the Wnt3a-producing cell line (Willert et al. 2003) with or without the Porcupine inhibitor IWP2 (B. Chen et al. 2009, S. Chen et al. 2009). After treatment with the conditioned media, the L-cells were washed with warm calcium and magnesium free phosphate buffered saline (PBS⁼) and then scraped from the well in to ~1 ml of PBS⁼. The cells were then pelleted at 1200 rpm for 2min and resuspended in 50 µl of PBS⁼, followed immediately by the addition of hot (≥95°C) 2x Laemmli Sample Buffer (Bio-Rad Laboratories, 161-0737) with 0.5M β-mercaptoethanol and then incubated at ≥95°C for 5 min. The whole cell lysate protein samples were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the separated proteins transferred to a nitrocellulose membrane (Hybond C-Extra, Amersham, RPN303E). After transfer, the membrane was blocked with 5% (w/v) skim milk powder in Tris buffered saline (TBS) with 0.1% Tween-20 (TBS-T) for 1hr at room temperature, rinsed and incubated overnight in primary antibody (mouse anti-β-catenin, BD Transduction Labs, 610154) at 4°C. Following the overnight

incubation, the primary antibody was washed off with TBS-T and the bound antibody detected with anti-mouse-HRP (DAKO, P0161) and Enhanced Chemiluminescence (ECL) [Clarity Western ECL Substrates; Luminol/enhancer reagent and Peroxide reagent (Bio-Rad, 1705060)].

Immunofluorescence staining

To stain LIM1863-*Mph* during MET, detached monolayer cells were seeded at $5 \times 10^3/\text{cm}^2$ in 2-chamber slides (Lab-Tek, 177429) as described (Vincan et al. 2008) and given the indicated treatments. Following treatment, the growth medium was removed and the cells were washed twice in PBS⁺⁺ (PBS with Mg⁺⁺/Ca⁺⁺) and fixed *in situ* with methanol at -20°C for 20 min. The cells were incubated for 1hr in 5% FCS in 0.2% PBST to block nonspecific binding of the antibodies. The primary antibody was diluted in 5% FCS in 0.05% PBST (PBS + 0.05% Triton X-100), and left overnight at 4°C. After washing, the cells were incubated in the secondary antibody for 1hr at room temperature and then DAPI (4'-6-Diamidino-2-phenylindole) was added to stain the nuclei. The cells were mounted in 70% glycerol for confocal microscopy. The primary antibodies used were mouse anti-E-cadherin (BD Transduction Labs, 610181) and mouse anti-ZO-1 (Invitrogen, 339100). Secondary antibody was Alexa anti-mouse 568. Detection of the staining for the chamber slides was captured on a Leica LAS AF SP5 microscope using a 20× HCX Plan Apo NA 0.7 IMM objective.

Free-floating organoids were fixed in 4.0% paraformaldehyde in PBS⁼ and permeabilized with 0.2% PBST (PBS⁼ with 0.2% Triton X-100). Primary antibody was E-cadherin and rabbit anti-Wnt2B (abcam, ab50575). Secondary antibodies were Alexa anti-mouse 594 and Alexa anti-rabbit 488. Staining of the free-floating organoids was captured using the Zeiss LSM Meta confocal.

Z-stacks of images was collected and processed in ImageJ v1.43u, then finalized in Adobe Photoshop CS4 v11.0.2 to generate the final image. All images from an experiment were processed in the same way.

RNA extraction, cDNA synthesis and quantitative PCR

Gene expression by qRT-PCR (quantitative reverse transcriptase polymerase chain reaction) was performed as we previously described (Vincan et al. 2005). Briefly, following

treatment, the cells were harvested in TRIzol (Invitrogen 15596-018) and total RNA was purified and DNase treated using RNeasy mini-prep columns (Qiagen 74104). cDNA was synthesized from 4µg of total RNA using anchored oligodT primers (Promega) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega M1705). Quantitative PCR on the cDNA samples was performed using SYBR green (Invitrogen 4309155) and ABI PRISM 7500 Sequence Detector (Applied Biosystems). The housekeeping gene hydroxymethyl-bilane synthase (HMBS) (Vandesompele et al. 2002) was used to calculate the fold change ($2^{\Delta\Delta CT}$ method) as described (Vincan et al. 2005). Primer sequences are available on request.

For the array analysis, cDNA (four separate monolayer and organoid populations) was synthesized as above and then assayed on the Human Wnt Signaling Pathway Array (Qiagen, PAHS-043YA). HMBS expression was determined by qPCR as above and used to calculate the fold change ($2^{\Delta\Delta CT}$ method) as described (Vincan et al. 2005).

Statistical analysis

Data are expressed as mean \pm SEM, where mean represents number of experiments (three or more). Statistical tests used are Student's t test or Mann-Whitney with Prism6 (GraphPad software) where P values of ≤ 0.05 were considered significant.

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